PATHOGENS AND GLOBAL HEALTH
2020, VOL. 114, NO. 4, 194–204
https://doi.org/10.1080/20477724.2020.1755551

Taylor & Francis
Taylor & Francis Group

Curcuma longa ethanol extract and Curcumin inhibit the growth of *Acanthamoeba triangularis* trophozoites and cysts isolated from water reservoirs at Walailak University, Thailand

Watcharapong Mitsuwan[a], Chooseel Bunsuwansakul[a], Theodore Ebenezer Leonard[a,b], Sawanya Laohaprapanon[c], Kruawan Hounkong[d], Kingkan Bunluepuech[e], Chalermpon Kaewjai[f], Tooba Mahboob[g], Chandramathi Sumudi Raju[g], Mahaveer Dhobi[h], Maria de Lourdes Pereira[i], Muhammad Nawaz[j], Christophe Wiart[k], Abolghasem Siyadatpanah[l], Roghayeh Norouzi[m] and Veeranoot Nissapatorn[a]

[a]School of Allied Health Sciences, Southeast Asia Water Team (SEA Water Team) and World Union for Herbal Drug Discovery (WUHeDD), Walailak University, Nakhon Si Thammarat, Thailand; [b]Faculty of Pharmacy, Indonesia International Institute for Life Sciences, Jakarta, Indonesia; [c]School of Public Health, Walailak University, Nakorn Si Thammarat, Thailand; [d]Faculty of Medicine, Princess of Naradhiwas University, Narathiwat, Thailand; [e]School of Allied Health Sciences and Research Excellence Center for Innovation and Health Products (RECIHP), Walailak University, Nakhon Si Thammarat, Thailand; [f]Faculty of Medical Technology, Rangsit University, Pathum Thani, Thailand; [g]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia; [h]Department of Pharmacognosy and Phytochemistry, Delhi Pharmaceutical Sciences and Research University, Delhi, India; [i]Department of Medical Sciences and CICECO-Aveiro Institute of Materials, University of Aveiro, Aveiro, Portugal; [j]Department of Nano-Medicine Research, Institute for Research and Medical Consultations, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia; [k]School of Pharmacy, Nottingham University, Selangor, Malaysia; [l]Ferdows School of Paramedical and Health, Birjand University of Medical Sciences, Birjand, Iran; [m]Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

ABSTRACT

Curcuma longa (*C. longa*) rhizome extract has been traditionally used to treat many infections. Curcumin, a pure compound isolated from the plant, has been documented to possess a wide spectrum of pharmacological effects. The present study aimed to investigate the effects of Thai medicinal plant extracts including *C. longa* extract and Curcumin on *Acanthamoeba triangularis*, a causative agent of human *Acanthamoeba* keratitis. The parasite was isolated from the recreational reservoir at Walailak University, Thailand. The organism was identified as *A. triangularis* using morphology and 18S rDNA nucleotide sequences. The pathogen was tested for their susceptibility to ethanol extracts of Thai medicinal plants based on eye infection treatment. The ethanol *C. longa* extract showed the strongest anti-*Acanthamoeba* activity against both the trophozoites and cysts, followed by *Coscinium fenestratum, Coccinia grandis*, and *Acmella oleracea* extracts, respectively. After 24 h, 95% reduction of trophozoite viability was significantly decreased following the treatment with *C. longa* extract at 125 µg/mL, compared with the control ($P < 0.05$). The extract at 1,000 µg/mL inhibited 90% viability of *Acanthamoeba* cyst within 24 h, compared with the control. It was found that the cysts treated with *C. longa* extract at 500 µg/mL demonstrated abnormal shape after 24 h. The MIC values of *C. longa* extract and Curcumin against the trophozoites were 125 and 62.5 µg/mL, respectively. While the MICs of the extract and curcumin against the cysts were 500 and 1,000 µg/mL, respectively. The results suggested the potential medicinal benefits of *C. longa* extract and Curcumin as the alternative treatment of *Acanthamoeba* infections.

KEYWORDS

Acanthamoeba triangularis; Anti-*Acanthamoeba* activity; *Curcuma longa* extract; Curcumin; Cysts; Trophozoites

1. Introduction

Free-living amebae belonging to the genus *Acanthamoeba* are protozoa ubiquitously in nature such as water and soil. The protozoa are causative agents of several diseases including granulomatous amebic encephalitis [1–3] and amebic keratitis [4]. The occurrence of *Acanthamoeba* keratitis in contact lens users can cause severe vision loss and complete blindness [5]. In addition, the infection caused by the organism is severe in immunocompromised patients. The parasite has two stages of growth including trophozoite and cyst. Trophozoite is a vegetative amoeba form moving by amoeboid locomotion. Cyst form is dormant stage that survives in harsh environment conditions such as lack of nutrients. *Acanthamoeba* cysts are classified into three groups including astronyxids, polyphagids, and culbertsonids [6]. The cysts contain two strong layers of cyst wall including ectocyst and endocyst walls. *Acanthamoeba* cysts have been reported to resist to antimicrobial substances [7,8]. Therefore, the treatment of *Acanthamoeba* infections is difficult due to its double-walled cyst layers.

CONTACT Veeranoot Nissapatorn veeranoot.ni@wu.ac.th School of Allied Health Sciences, Research Excellence Center for Innovation and Health Products (RECIHP), Walailak University, Nakhon Si Thammarat, Thailand

This article has been republished with minor changes. These changes do not impact the academic content of the article.

In an attempt to overcome the infections caused by *Acanthamoeba* spp., targeting natural compounds for their anti-parasitic properties could be applied for the treatment. Plant-derived compounds have been focused to treat *Acanthamoeba* infection due to the activities of their bio-active molecules. *Trigonella foenum graecum* seeds decreased the numbers of viable trophozoites and cysts of a clinical strain of *Acanthamoeba* genotype T4 [9]. *Artemisia annua* extract and its pure compound, artemisinin, inhibited *Acanthamoeba* keratitis, genotype T4 *in vitro* and *in vivo* study [10]. So far, there was no similar study done in southern Thailand.

The present study is therefore highlighted on the ethanol extracts of Thai medicinal plants based on eye infection treatment. The objective of this study was to investigate the anti-*Acanthamoeba* activity of *Curcuma longa* extract and Curcumin compound against a pathogenic free-living amoeba (FLA) of *Acanthamoeba triangularis* isolated from Walailak University reservoirs.

2. Materials and methods

2.1. Water sample

Three liters grabbed water samples of 8 points were collected from two reservoirs including recreational and botanical garden reservoirs at Walailak University, Nakhon Si Thammarat, Thailand. Decimal degrees and water temperature data were recorded at sampling sites. In addition, pH record with the pH meter (Mettler Toledo, Ohio, USA), physico-chemical properties (SevenCompact conductivity, Mettler Toledo, Ohio, USA), Field water quality kits (Mahidol University, Bangkok, Thailand), Eutech TN-100 waterproof turbidimeter (Thermo-Scientific, Bartlesville, USA), Color test kit (Hach, Colorado, USA), and 3 M™ Petrifilm™ *E. coli*/Coliform count plate 6404 (Emerald Scientific, CA, USA) were analyzed. Two liters of the samples were filtered with vacuum pump (Rocker 811, New Taipei City, Taiwan) through sterile gauze once and then 1.2 µM-pore size filter (Whatman, Buckinghamshire, United Kingdom). The debris on filter paper were scraped into 30 mL Page's saline solution (PAS) (Himedia, Maharashtra, India) and centrifuged at 3,000 × g for 30 min. The samples were re-suspended in 20 mL of PAS, and preserved at 4°C.

2.2. Free-living amoeba cultivation

The sediment from each water sample was dropped on moist non-nutrient agar (NNA: PAS and 1.5% biological agar, Oxoid) on plastic petri dish overlaid with autoclaved *Escherichia coli* (*E. coli*) ATCC25922 for 2–4 positions. The plates were incubated at room temperature. The plates were then observed daily using an inverted microscope (Nikon ECLIPSE TE2000-S, Tokyo, Japan) during incubation for at least 2 weeks or until all trophozoites moved out from sediment and formed into cysts. Cyst-like morphology was sub-cultured by transferring upside down <1 cm^2 agar piece into new NNA plus autoclaved *E. coli* lawn until homogenous morphology was observed. Each *Acanthamoeba* spp. clone was classified into three groups based on Pussard and Pons criteria as described [11].

For the anti-*Acanthamoeba* activity of medicinal plant extracts, parasite samples were directly inoculated on NNA plates seeded with the suspension of *E. coli* cells. The trophozoites were observed after 48–72 h of incubation at room temperature. The amoeba cells on the culture plates were scraped by sterile cell scraper in Page's Saline solution. The cells were washed twice with Page's saline solution and centrifuged at 3,000 × g for 5 min. The trophozoite viability was investigated using a trypan blue exclusion assay. The trophozoites were adjusted to a final concentration of 2×10^5 trophozoites/mL. The cysts were harvested when the cultures were incubated in 1 week. The preparation and viability of the cysts were determined by the protocol described above.

2.3. Molecular and phylogenetic analysis

At least 2-mL PBS (pH 7.0) were rinsed on culture on NNA and scraped to harvest trophozoites into microtubes. Trophozoite suspension was centrifuged at 1,500 rpm for 5 min. The supernatant was removed before adding 1 mL of PBS to wash. DNA was extracted using Zymo kit (Zymo Research, CA, USA). PCR was conducted to confirm *Acanthamoeba* genus by Primer JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′) [12] by Thermocycler (Eppendorf, Hamburg, Germany). The reaction mix (PCR Biosysems, London, UK) was adapted in 25 µL per reaction. PCR conditions include 1 min of 95°C for initial denaturation, 40 cycles: 15 s of 95°C for denaturation, 15 s of 60°C for annealing, and 15 s of 72°C for extension, 15 s of 72°C for a final extension, and 10°C for cooling. Amplicon size is near 500 bps, purified (Geneaid, New Taipei City, Taiwan), and sequenced (Macrogen, Seoul, Korea) to collect phylogenetic data on ASA.A1 of 18S rDNA. Nucleotide sequences were BLASTed on NCBI database and analyzed to create a phylogenetic tree to identify species as described [13].

2.4. Preparation of plant extracts

A total of 10 medicinal plants claimed to act as agents that cured eye infection or parasite infection were used and presented in Table 1. The plant materials were dried and extracted with 95% ethanol. The solvent was evaporated under reduced pressure. Curcumin, a pure compound isolated from *C. longa* rhizome, was used. The extracts and its pure

Table 1. Thai medicinal plants used in this study.

Botanical name	Family	Past used
Acmella oleracea (L.) *R.K. Jansen*	Asteraceae	Stem
Andrographis paniculata (Burm.f.) Wall. ex Nees	Acanthaceae	Leaf & stem
Azadirachta indica A. Juss. var. siamensis Valeton	Meliaceae	Leaf
Coccinia grandis (L.) Voigt	Cucurbitaceae	Vine
Coscinium fenestratum (Gaertn.) Colebr.	Menispermaceae	Rhizome
Curcuma longa L.	Zingiberaceae	Rhizome
Ipomoea aquatica Forssk.	Convolvulaceae	Leaf & Stem
Psidium guajava L.	Myrtaceae	Leaf
Syzygium aromaticum (L.) Merr. & Perry	Myrtaceae	Clove
Tamarindus indica L.	Fabaceae	Seed

compound were dissolved in 100% dimethyl sulfoxide (DMSO) and stored at 4°C.

2.5. Anti-*Acanthamoeba* activity of the plant extract against *Acanthamoeba* spp

Anti-*Acanthamoeba* activity of the extracts against the trophozoites and cysts of *Acanthamoeba* spp. was determined as described [14] with minor modification. The trophozoites and cysts were cultured as described above. Each of trophozoites and cysts was adjusted to a final concentration of 2×10^5 cells/mL. One hundred microliters of the parasite suspension were added into 96 well microtiter plates, containing 100 µL of serially diluted extract. The plates were incubated at room temperature for 24 h. One percent DMSO was included as a negative control. Inhibitory activity was carried out using a trypan blue exclusion assay. The relative percentage of parasite viability was defined as (mean of the treated parasite/mean of the control) × 100. Meanwhile, the morphology of the trophozoite and cyst forms of *A. triangularis* after treatment with *C. longa* extract was investigated preliminarily by inverted microscopy.

2.6. Determination of minimal inhibitory concentration of *C. longa* extract and Curcumin against *A. triangularis*

Minimal inhibitory concentration (MIC) of *C. longa* extract and Curcumin against *A. triangularis* was investigated by broth microdilution method as described [15]. The trophozoites and cysts were cultured as described above. Each of trophozoites and cysts was adjusted to a final concentration of 2×10^5 cells/mL. A total of 100 µL of the parasite suspension were added into 96 well microtiter plates, containing 100 µL of serially diluted *C. longa* extract and/or Curcumin. Then, 1% DMSO and Chlorhexidine were included as a negative and positive control, respectively. The plates were incubated at room temperature for 24 h. The MIC values were defined as the lowest concentration that >90% growth inhibition of the zoites and cysts, when compared with the negative control.

2.7. Scanning electron microscopy

The morphology of *A. triangularis* cysts after treatment with *C. longa* extract and Curcumin was observed in Scanning electron microscopy (SEM). The cysts were treated with different concentrations of the extract and Curcumin in a 24-well plate with a sterile circular glass coverslip. After 24-h incubation, the discs were washed three times with PBS. The samples were fixed with 2.5% glutaraldehyde in PBS for 24 h and subsequently washed with PBS. The specimens were dehydrated in a series of graded ethanol (25–100%), mounted on aluminum stubs, and allowed to dry using a critical point dryer. The samples were then coated with gold particles and the morphology of *Acanthamoeba* cysts after treatment was subsequently examined under SEM.

2.8. Statistical analysis

The experiments were performed in triplicate. All data were recorded and entered using the statistical package software (SPSS Inc. Chicago, IL, USA). The data were expressed as mean ± SD. Statistical analysis was analyzed by the two-tailed unpaired Student's t-test. In all analyzes, $P < 0.05$ were considered the statistically significant difference.

3. Results

3. 1. Isolation and identification of free living *Acanthamoeba*

A total of 32 water samples of each 8 points from 2 reservoirs including recreational and botanical garden reservoirs at Walailak University, Nakhon Si Thammarat, Thailand were collected (Figure 1). The results demonstrated that 32 samples of environmental water were contaminated with *A. triangularis* using microscopic examination. The samples were recorded as A1.1, A1.2, A2.1, A2.2, A3.1, A3.2, A4.1, A4.2, A5.1, A5.2, A6.1, A6.2, A7.1, A7.2, A8.1, and, A8.2. The samples collected from botanical garden reservoirs were named as B1.1, B1.2, B2.1, B2.2, B3.1, B3.2, B4.1, B4.2, B5.1, B5.2, B6.1, B6.2, B7.1, B7.2, B8.1, and, B8.2. Trophozoites (Figure 2(a)) and cysts (Figure 2(b)) were observed from all the samples within 1–2 weeks. Three groups of *Acanthamoeba* cysts including Astronyxids (Figure 3(a)), Polyphagids (Figure 3 (b)), and Culbertsonids (Figure 3(c)) were classified base on cyst morphology. Cysts from the sample no. A3.1

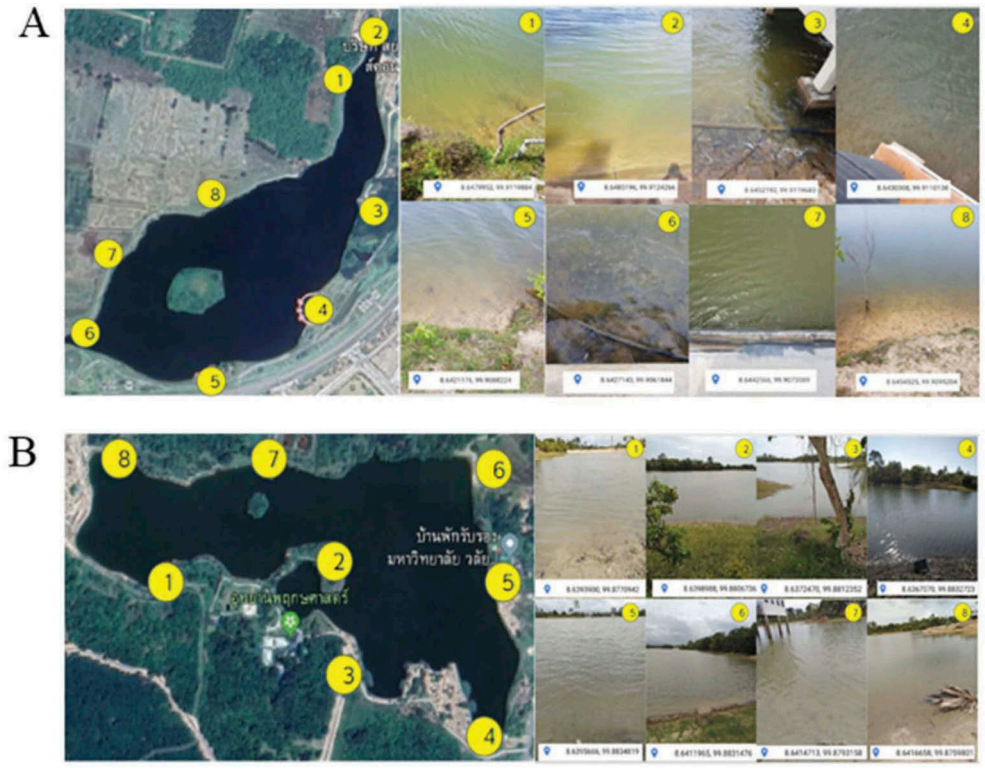

Figure 1. Map showing the area of water collection. A is recreational reservoir while B is botanical garden reservoir at Walailak university, Nakhon Si Thammarat, Thailand. The water samples were collected from 8 points of each the reservoirs. The large images are the aerial view of the ponds water. The small images are the sampling location.

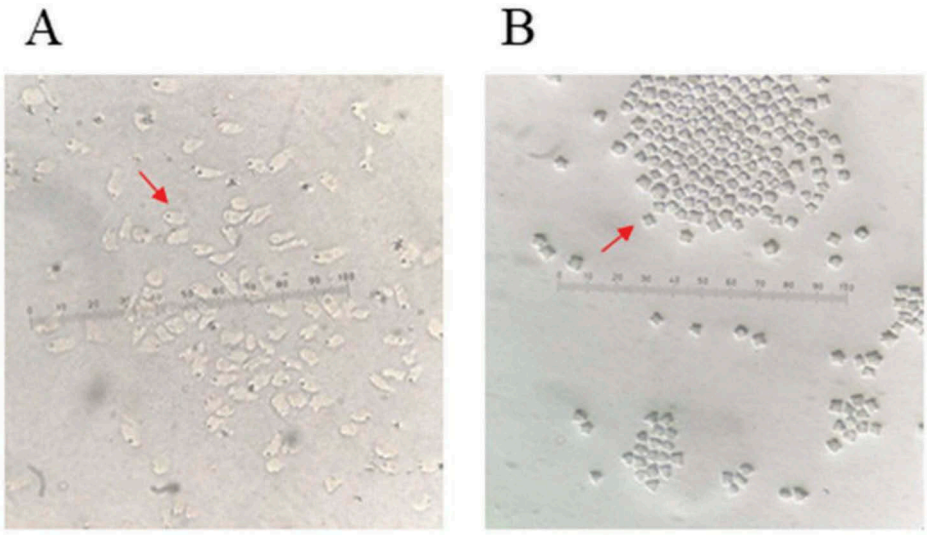

Figure 2. *Acanthamoeba* trophozoites (a) and cysts (b) found in recreational and botanical garden reservoirs. Magnification of 400X.

isolated from recreational reservoirs were chosen for further study because they showed a high amount of the cells.

3. 2. *Molecular identification of Acanthamoeba triangularis*

The cyst from the sample no. A3.1 was further identified using morphology and 18S rDNA nucleotide sequences. The trophozoite form and cyst form of the parasite was shown in Figures 4 (b) and 2(c), respectively. An isolated free-living amoeba cyst has thin ectocyst and triangular or four-pointed star endocyst and with 12–13 µM in size which is classified in group II *Acanthamoeba* spp. cyst morphology by Passard and Pons's criteria. PCR analysis of the isolate was conducted and a specific 500 bp band was expectable on an

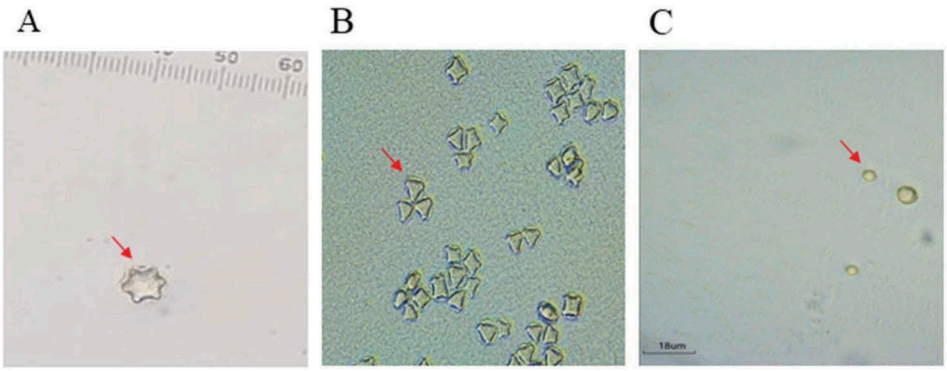

Figure 3. Microphotographs of isolated *Acanthamoeba* cysts including Astronyxids (a), Polyphagids (b), and Culbertsonids (c).

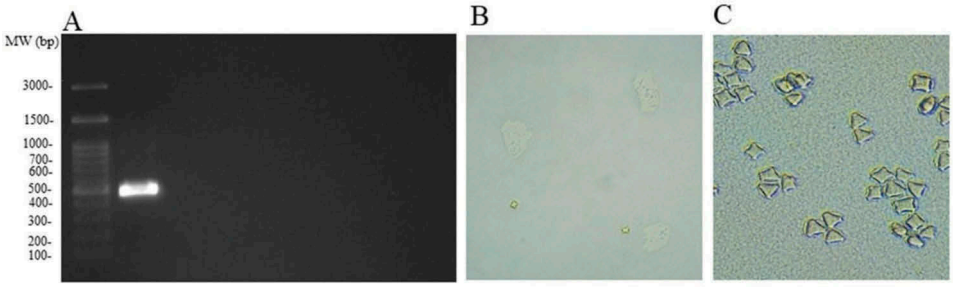

Figure 4. Identification of *Acanthamoeba triangularis* using molecular technique (a) and morphology (b and c). Agarose gel electrophoresis of the amplified products of *Acanthamoeba*-DNA extracted from the sample no. A3.1(a). *Acanthamoeba triangularis* trophozoites (b) and cysts (c).

agarose gel (Figure 4(a)). It was speculated that the isolate was positive for *Acanthamoeba* on PCR using genus-specific primers (JDP1 and JDP2). To identify the species, the 18S rRNA gene sequence of the parasite was further compared with *Acanthamoeba* sequences in the GenBank database. It was found that the parasitic isolate belonged to *A. triangularis* with a 100% similarity of 373 bps. Figure 5 shows the nucleotide sequence of the isolate *A. triangularis*. The genome sequences of the isolates were submitted to the GenBank database under the accession numbers KX231518.

```
Isolated      1 ----------------------------AGCAGGCAGATCCAATTCTCTGCCACCGAATAC
KX232518.1    1 TTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAATTCTCTGCCACCGAATAC

Isolated     34 ATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCG
KX232518.1   61 ATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGGTTTTGGCAGCG

Isolated     94 CGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAG
KX232518.1  121 CGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAG

Isolated    154 GTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTT
KX232518.1  181 GTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTT

Isolated    214 CATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAAC
KX232518.1  241 CATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAAC

Isolated    274 CATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCG
KX232518.1  301 CATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGTGCG

Isolated    334 GTCGTTCTTGGCGTCGGTTTCGGCCGGCGCGGGAGCGGCT--------------------
KX232518.1  361 GTCGTTCTTGGCGTCGGTTTCGGCCGGCGCGGGAGCGGCTTAGCCCGGTGGCACCGGTGA

Isolated        ------------------------
KX232518.1  421 ATGACTCCCTAGCAGCTTGTGAGA
```

Figure 5. Gene sequence of 18S rRNA *Acanthamoeba triangularis* isolate no. A3.1. The results exhibited that 373 bp of phylogenetically informative ASA.A1 sequence of 18S rDNA are identical to *Acanthamoeba triangularis* accession number KX231518.

3.3. Anti-*Acanthamoeba* activity of Thai medicinal plant extracts against *Acanthamoeba* trophozoites and cysts

Anti-*Acanthamoeba* activity of 10 ethanol plant extracts (Table 1) against *Acanthamoeba* trophozoites and cysts was investigated. All the extracts significantly inhibited the growth of *Acanthamoeba* trophozoites, compared with the control ($P < 0.05$) (Figure 6). *C. longa* extract exhibited the strongest anti-trophozoite activity against *Acanthamoeba*, followed by *Coscinium fenestratum, Coccinia grandis, Acmella oleracea*, and *Ipomoea aquatica*, respectively. After 24 h, a 95% reduction of trophozoite viability was significantly decreased following the treatment with *C. longa* extract at 125 µg/mL when compared with the control. Furthermore, an 80% reduction of the trophozoites was observed when the microorganisms was treated with *C. fenestratum, C. grandis, A. oleracea*, and *I. aquatica* extracts at 125–250 µg/mL, compared with the negative control.

The medicinal plant extracts were further determined for their anti-*Acanthamoeba* activity against *Acanthamoeba* cysts. *C. longa* showed the strongest anti-*Acanthamoeba* activity against *Acanthamoeba* cysts, followed by *C. fenestratum* extracts (Figure 7). After 24 h, a significant decrease in a 90% viability of

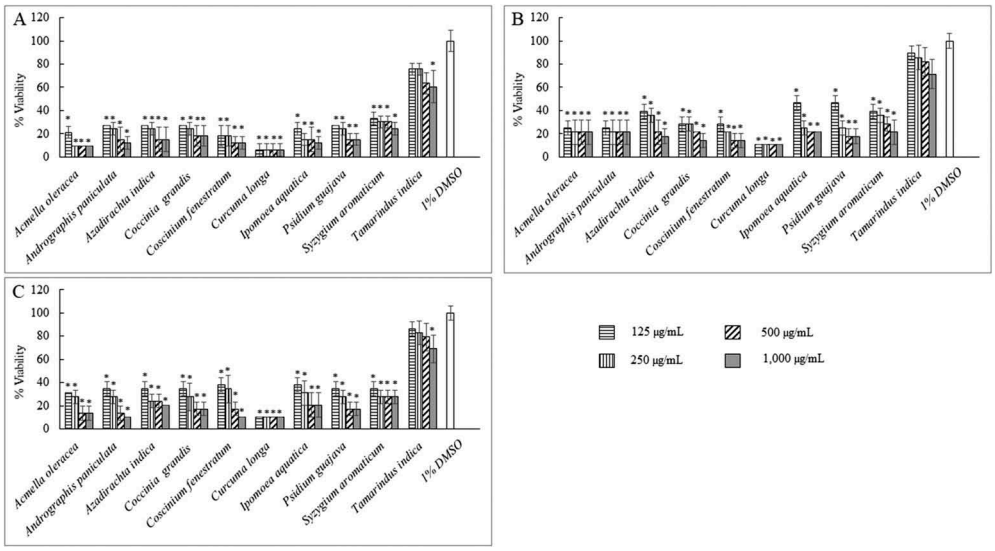

Figure 6. Effects of medicinal plant extracts on viability of *Acanthamoeba* spp. trophozoites. Cells were treated with different concentrations of the extracts, incubated at room temperature for 24 h (a), 48 h (b), and 72 h (c). Inhibitory activity was carried out using trypan blue exclusion assay. 1% DMSO was used as negative control. The relative percentage of cyst viability was defined as (mean of the treated cysts/mean of the control) × 100 (* significant difference; $P < 0.05$).

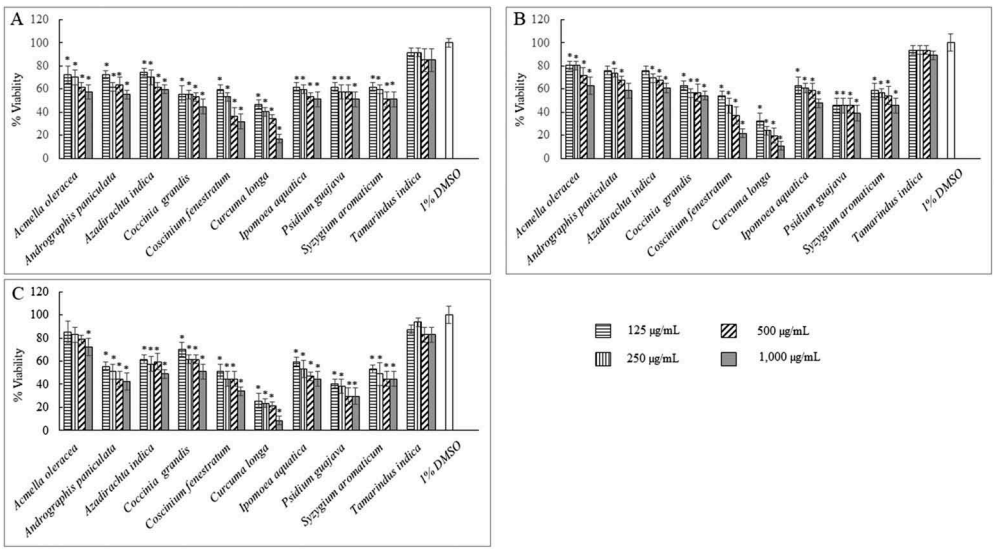

Figure 7. Effects of medicinal plant extracts on viability of *Acanthamoeba* spp. cysts. Cells were treated with different concentrations of the extracts, incubated at room temperature for 24 h (a), 48 h (b), and 72 h (c). Inhibitory activity was carried out using trypan blue exclusion assay. 1% DMSO was used as negative control. The relative percentage of cyst viability was defined as (mean of the treated cysts/mean of the control) × 100 (* significant difference; $P < 0.05$).

Acanthamoeba cysts was detected following the treatment with *C. longa* extract at 1,000 µg/mL, compared with the control ($P < 0.05$). The activity of *C. longa* extract was concentration and time-dependent manner observed after 24, 48, and 72 h. Therefore, *C. longa* extract was chosen for the further studies.

3.4. Effects of *C. longa* extract on *Acanthamoeba* trophozoites and cysts

The effects of *C. longa* extract on *A. triangularis* cells were determined by an inverted microscope. After 24 h, both trophozoite and cyst form of *A. triangularis* were observed in the negative control while the cyst form was detected in the treatment groups (Figure 8). In addition, the triangle shape of *A. triangularis* cysts was observed in non-treated cells (Figure 9). It was found that the cysts treated with the extract at 500 µg/mL demonstrated deformity (abnormal shape) after the initial 24 h.

3.5. Minimal inhibitory concentration (MIC) of *C. longa* extract and Curcumin against *A. triangularis*

The MIC values of *C. longa* extract and Curcumin against *A. triangularis* trophozoites and cysts are presented in Table 2. The extract demonstrated strong anti-*Acanthamoeba* activity against *A. triangularis* trophozoites and cysts with the MIC values of 125 and 1,000 µg/mL, respectively. The MIC values of Curcumin against the trophozoites and cysts were 62.5 and 500 µg/mL, respectively.

3.6. Morphology of *A. triangularis* cysts after treatment with *C. longa* extract and Curcumin

The morphology of *A. triangularis* cysts after treatment with *C. longa* extract and pure Curcumin compound was observed in SEM. The cysts, triangular in shape and smooth surface, were observed in the control (Figure 10 (a–c)). It was found that the cysts treated with the extract

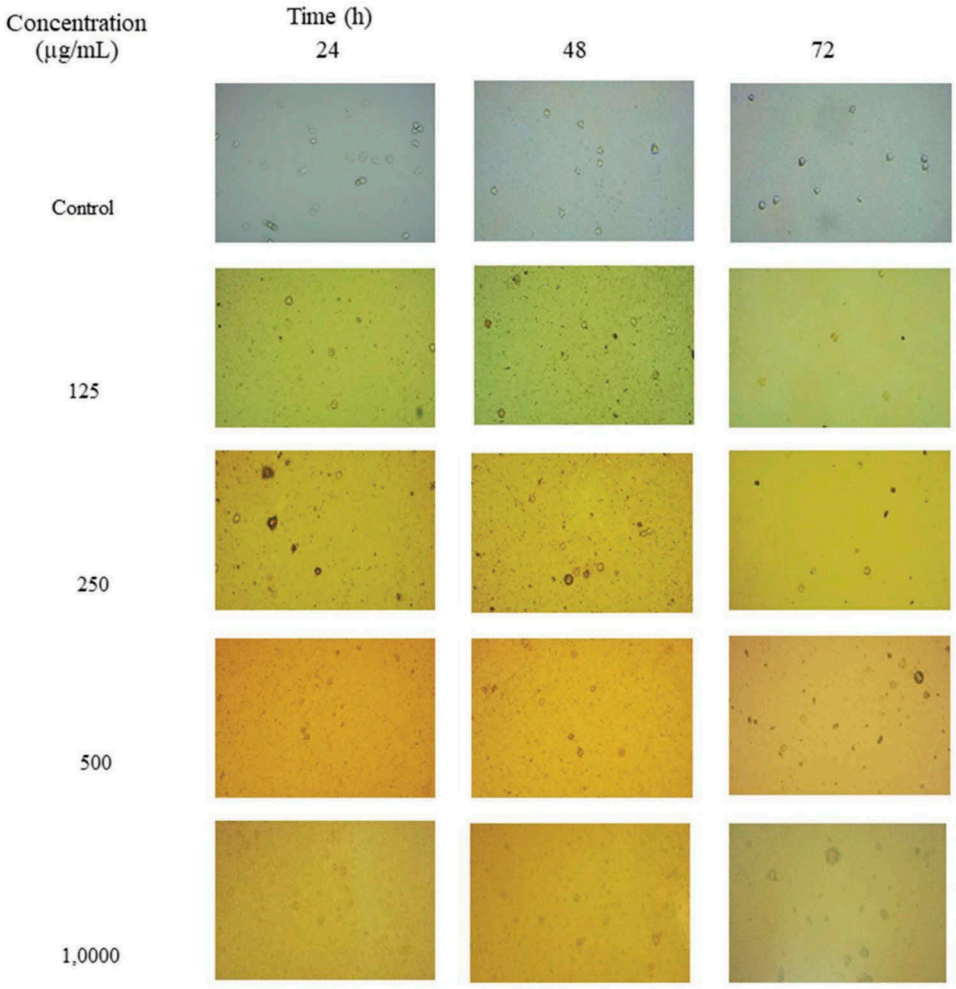

Figure 8. Effects of *Curcuma longa* extract on *Acanthamoeba* spp. trophozoites. Cells were treated with the extract at different concentrations, incubated for 24, 48, and 72 h. One percent DMSO was included as negative control. Images of the treated cysts were observed by inverted microscope (200X). After 24 h, trophozoite form of *A. triangularis* was observed in the negative control while the cyst form was detected in the treatment groups.

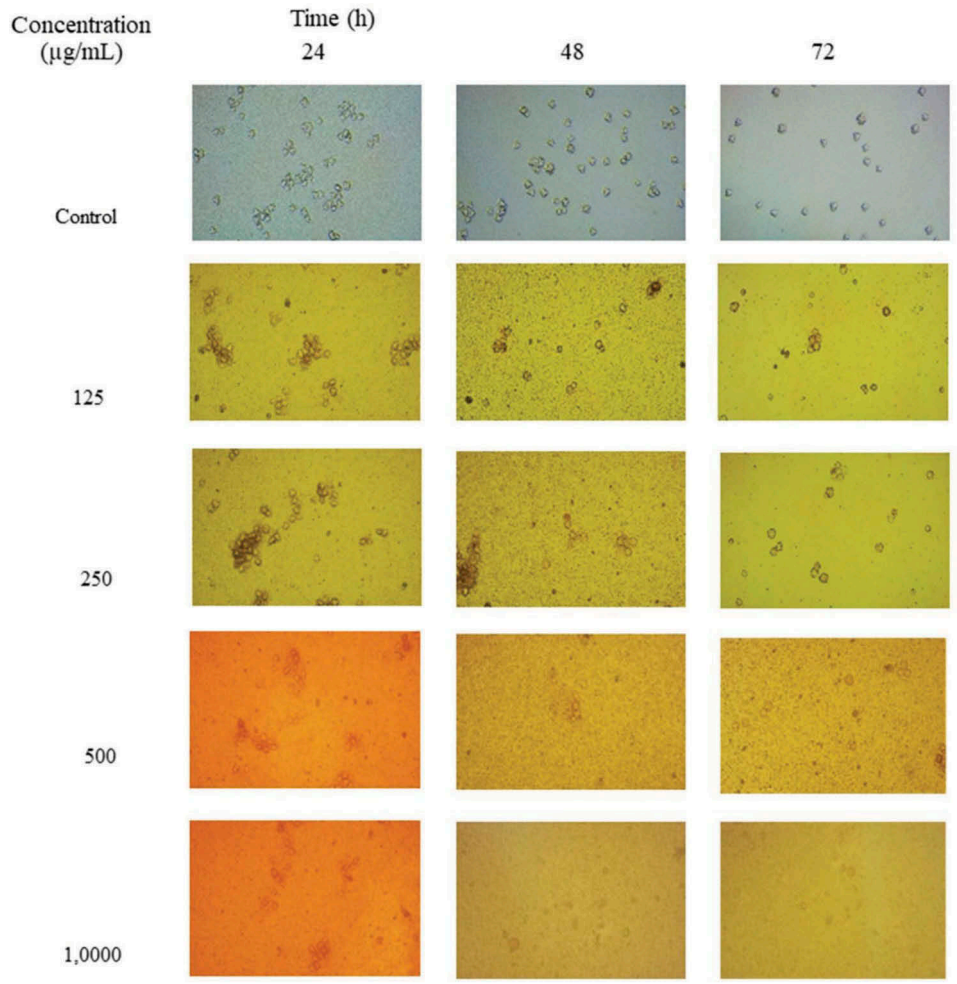

Figure 9. Effects of *Curcuma longa* extract on *Acanthamoeba* spp. cysts. Cells were treated with the extract at different concentrations, incubated for 24, 48, and 72 h. One percent DMSO was included as negative control. Images of the treated cysts were observed by inverted microscope (200X). Triangle shape of *A. triangularis* cysts was observed in non-treated cells. It was found that the cysts treated with the extract at high concentration demonstrated abnormal shape after 24 h.

Table 2. Minimal inhibitory concentration (MIC) of *C. longa* extract and Curcumin against *Acanthamoeba triangularis* trophozoites and cysts.

	MIC (µg/mL)	
Antimicrobial agents	Trophozoites	Cysts
C. longa extract	125	1,000
Curcumin	62.5	500
Chlorhexidine	6.25	25

(Figure 10 (d–i)) and Curcumin (Figure 10(j–o)) at 0.5 and 1× MIC showed deformity in the form of shrinkage shapes, when compared with the control. These shrink cysts were also observed when challenged with the extract and the pure compound at 0.5× MIC.

4. Discussion

Acanthamoeba spp. are the main cause of keratitis in contact lens users [5]. The present study interestingly detected the occurrence of *Acanthamoeba* spp. in different pond water samples, as also reported in a previous study [16]. Our finding further identified the species of *Acanthamoeba* using the 18S rRNA gene sequence and *A. triangularis* was subsequently confirmed with 100% similarity, compared to the GenBank sequence database. The sensitive high-performance molecular-based method should therefore be recommended for the species identification of this free-living amoeba [17].

The present study is focused on anti-*Acanthamoeba* activity of ethanol extracts of Thai medicinal plants based on eye infection treatment. The results demonstrated that *C. longa* extract showed the strongest anti-*Acanthamoeba* activity against both the trophozoites and cysts. Interestingly, *C. longa* essential oil has been reported of inhibiting *Acanthamoeba* spp. trophozoites but not for the cyst form [18]. Inhibition of *A. castellanii* cysts by *C. longa* ethanol extract has been reported [19]. In addition, *C. longa* extract possessed other anti-parasitic activities against various pathogenic organisms including *Ascaridia galli* [20], *Haemonchus* larval stage [21], *Schistosoma mansoni* [22], and *Trypanosoma* spp [23].

Curcumin, the main bio-active compound, is isolated from *C. longa* that serves for various therapeutic and

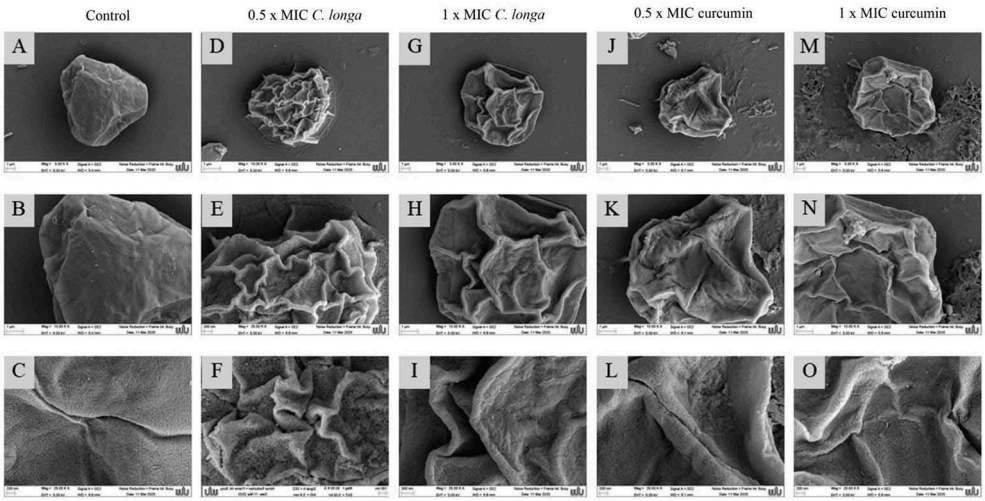

Figure 10. Morphology of *A. triangularis* cysts after treatment with *C. longa* extract and Curcumin. The cells were treated with the extract and Curcumin at different concentrations. One percent DMSO was used as negative control. Morphology of the cysts was observed by SEM. Magnification: A, D, G, J, M = 5,000X; B, E, H, K, N = 10, 000X; C, F, I, L, O = 25,000X.

preventive purposes. The present study demonstrated that the MIC values of Curcumin was two times lower than those of the *C. longa* extract. It has been highlighted that the compound is a bio-active compound presented in the plant species [24]. It has also been reported that dimethoxy Curcumin, curcuminoids presented in *C. longa* rhizomes, exhibited significant amoebicidal effects against *A. castellanii* [25]. Moreover, Curcumin possesses biological activity such as anti-inflammatory, antioxidant, antiviral, and antibacterial properties [26].

Interestingly, cysts treated with *C. longa* extract and Curcumin demonstrated shrinkage in shapes, when compared with the control. Generally, it has been reported that the cyst stage of *Acanthamoeba* spp. is resistant to various environmental stressors, such as extreme conditions (starvation, temperatures, pH, osmolarity, irradiation, and drugs) [27,28], and antimicrobial substances [7,8]. Our study clearly supports the role of stress management, demonstrating the efficacy of this plant extract and its pure compound in *A. triangularis* with the deformity of cysts after treatment, as shown in Figure 10.

Overall, the results demonstrated that *C. longa* extract and Curcumin showed the strongest anti-*Acanthamoeba* activity against *A. triangularis* compared with the tested plant extracts. However, the cysts were not completely inhibited by the extract due to the strong layers of the cyst walls. Diamidines and biguanides are often the first-line therapy for *Acanthamoeba* keratitis [29]. In addition, the treatment of this disease requires several months due to the stage of the cyst. Therefore, medical therapeutic intervention tends to produce more side effects in the long-term [30]. It was suggested that combination of currently available drugs and/or natural products could be used as an alternative strategy to kill *Acanthamoeba*. The anti-*Acanthamoeba* synergistic effect of chlorhexidine and cationic carbosilane dendrimers against the trophozoite and cyst forms of *A. polyphaga* has been reported [30]. Currently, the synthesis of nanoparticles using plant extracts has been reported as one of the ways to improve the efficacy of these compounds in destroying the cysts [31]. In addition, the aqueous extract of *Nigella sativa* in combination with chitosan nanoparticles has shown interestingly synergistic effects against *Acanthamoeba* keratitis [32]. To support this, additional studies on Curcumin are ongoing in our laboratories to validate the efficacy in the treatment of *Acanthamoeba* infection.

5. Conclusion

The results demonstrated that *C. longa* extract and Curcumin showed the strongest anti-*Acanthamoeba* activity against both the trophozoites and cysts. After 24 h, a 95% reduction of trophozoite viability was significantly decreased following the treatment with *C. longa* extract at 125 µg/mL, compared with the control. In addition, the extract at 1,000 µg/mL inhibited a 90% viability of *Acanthamoeba* cyst within 24 h when compared with the control. *C. longa* extract and Curcumin showed strong anti-*Acanthamoeba* activity against the zoites with low MIC values. The results suggested the potential medicinal benefits of the extract and Curcumin for the treatment of *Acanthamoeba* infections. It is also strongly recommended for further study on biofilms, proteomics, metabolomics as well as nano-preparation (in simple or combined forms) on Curcumin to see the potential source of this plant-based against *Acanthamoeba* infection in the near future.

Acknowledgments

This work was conducted under the project entitled of - Medicinal under-exploited Thai native plants against *Acanthamoeba, Leishmania donovani*, and *Plasmodium falciparum* – Toward South East Asia collaboration initiative supported by The Royal Patronage of Her Royal Highness Princess Maha Chakri Sirindhorn (Grant No. 040226) and CICECO-Aveiro Institute of Materials (UIDB/50011/2020 & UIDP/50011/2020). We are also grateful to the Research Institute of Health Science, Walailak University for the laboratory facilities.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by The Royal Patronage of Her Royal Highness Princess Maha Chakri Sirindhorn [Grant No. 040226].

References

[1] Rodríguez-Pérez EG, Escandón-Vargas K, Ancer AJ. Granulomatous amebic encephalitis caused by *Acanthamoeba* sp. in an immunocompetent Mexican adult. Rev Soc Bras Med Trop. 2017;50(3):432–433.

[2] Lee DC, Fiester SE, Madeline LA, et al. *Acanthamoeba* spp. and *Balamuthia mandrillaris* leading to fatal granulomatous amebic encephalitis. Forensic Sci Med Pathol. 2019;16(1):171–176.

[3] Kalra SK, Sharma P, Shyam K, et al. *Acanthamoeba* and its pathogenic role in granulomatous amebic encephalitis. Exp Parasitol. 2020;208:107788.

[4] Jercic MI, Aguayo C, Saldarriaga-Córdoba M, et al. Genotypic diversity of *Acanthamoeba* strains isolated from Chilean patients with *Acanthamoeba* keratitis. Parasit Vectors. 2019;12(1):58.

[5] Lee SM, Lee JE, Lee DI, et al. Adhesion of *Acanthamoeba* on cosmetic contact lenses. J Korean Med Sci. 2017;33(4):e26.

[6] Anwar A, Khan NA, Siddiqui R. Combating *Acanthamoeba* spp. cysts: what are the options? Parasit Vectors. 2018;11(1):26.

[7] Storey MV, Winiecka-Krusnell J, Ashbolt NJ, et al. The efficacy of heat and chlorine treatment against thermotolerant *Acanthamoebae* and *Legionellae*. Scand J Infect Dis. 2004;36(9):656–662.

[8] Coulon C, Collignon A, McDonnell G, et al. Resistance of *Acanthamoeba* cysts to disinfection treatments used in health care settings. J Clin Microbiol. 2010;48(8):2689–2697.

[9] Dodangeh S, Niyyati M, Kamalinejad M, et al. *In vitro* activity of *Trigonella foenum graecum* seeds against a clinical strain of *Acanthamoeba* genotype T4. Iran J Pharm Res. 2018;17(2):661–667.

[10] Derda M, Hadaś E, Cholewiński M, et al. *Artemisia annua* L. as a plant with potential use in the treatment of acanthamoebiasis. Parasitol Res. 2016;115(4):1635–1639.

[11] Pussard M, Pons R, Mas-Pons R. Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). Protistologica. 1997;13(4):557–598.

[12] Dykova I, Lom J, Schroeder-Diedrich JM, et al. *Acanthamoeba* strains isolated from organs of freshwater fishes. J Parasitol. 1999;85(6):1106–1113.

[13] Johnson M, Zaretskaya I, Raytselis Y, et al. NCBI BLAST: a better web interface. Nucleic Acids Res. 2008;36:W5–W9.

[14] Mahboob T, Azlan A-M, Shipton FN, et al. Acanthamoebicidal activity of periglaucine A and betulinic acid from *Pericampylus glaucus* (Lam.) Merr. *in vitro*. Exp Parasitol. 2017;183:160–166.

[15] Hounkong K, Sawangjaroen N, Kongyen W, et al. Anti-intestinal protozoan activities of 1-hydroxy-2 hydroxymethylanthraquinone from *Coptosapelta flavescens*. Asian Pac J Trop Dis. 2014;4(6):457–462.

[16] Manesh RM, Niyyati M, Yousefi HA, et al. Isolation of *Acanthamoeba* spp. from different water sources in Isfahan, central Iran, 2014. J Parasit Dis. 2016;40(4):1483–1486.

[17] Magnet A, Fenoy S, Galván A, et al. A year long study of the presence of free living amoeba in Spain. Water Res. 2013;47(19):6966–6972.

[18] Sanguan S, Wannasan A, Junkum A, et al. Screening for *in vitro* amoebicidal activity of plant essential oils against *Acanthamoeba* sp. Chiang Mai Med J. 2018;57(2):89–98.

[19] El-Sayed NM, Ismail KA, Ahmed SA-E-G, et al. *In vitro* amoebicidal activity of ethanol extracts of *Arachis hypogaea* L., *Curcuma longa* L. and *Pancratium maritimum* L. on *Acanthamoeba castellanii* cysts. Parasitol Res. 2012;110(5):1985–1992.

[20] Alrubaie AL. Effects of alcoholic extract of *Curcuma longa* on *Ascaridia* infestation affecting chicken. Indian J Exp Biol. 2015;53(7):452–456.

[21] Nasai NB, Abba Y, Abdullah FFJ, et al. *In vitro* larvicidal effects of ethanolic extract of *Curcuma longa* Linn. on *Haemonchus* larval stage. Vet World. 2016;9(4):417–420.

[22] Hussein A, Rashed S, El Hayawan I, et al. Evaluation of the anti- schistosomal effects of Turmeric (*Curcuma longa*) versus praziquantel in *Schistosoma mansoni* infected mice. Iran J Parasitol. 2017;12(4):587–596.

[23] Le TB, Beaufay C, Nghiem DT, et al. Evaluation of the anti-Trypanosomal activity of Vietnamese essential oils, with emphasis on *Curcuma longa* L. and its components. Molecules. 2019;24(6):1158.

[24] Agarwal A, Saxena PN. Curcumin, a polyphenol from the *Curcuma longa*, prevents mercuric chloride-induced liver damage through reversal of oxidative stress and biochemical changes. Abstract nr 4925. AACR. 2018;78(13).

[25] Aqeel Y, Iqbal J, Siddiqui R, et al. Anti-Acanthamoebic properties of resveratrol and demethoxycurcumin. Exp Parasitol. 2012;132(4):519–523.

[26] Aggarwal BB, Sung B. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends Pharmacol Sci. 2009;30(2):85–94.

[27] Mazur T, Hadaś E, Iwanicka I. The duration of the cyst stage and the viability and virulence of *Acanthamoeba* isolates. Trop Med Parasitol. 1995;46(2):106–108.

[28] Khan NA, Iqbal J. Siddiqui RJBri: stress management in cyst-forming free-living protists: programmed cell death and/or encystment. Biomed Res Int. 2015;2015:437534.

[29] Szentmáry N, Daas L, Shi L, et al. *Acanthamoeba* keratitis-clinical signs, differential diagnosis and treatment. J Curr Ophthalmol. 2019;31(1):16–23.
[30] Heredero-Bermejo I, Sanchez-Nieves J, Soliveri J, et al. *In vitro* anti-*Acanthamoeba* synergistic effect of chlorhexidineand cationic carbosilane dendrimers against both trophozoite and cyst forms. Int J Pharm. 2016;509(1–2):1–7.
[31] Mahboob T, Nawaz M, Tian-Chye T, et al. Preparation of poly (dl-lactide-co-glycolide) nanoparticles encapsulated with periglaucine A and betulinic acid for *in vitro* anti-*Acanthamoeba* and cytotoxicity activities. Pathogens. 2018;7(3):62.
[32] Elkadery AAS, Elsherif EA, Eldin HME, et al. Efficient therapeutic effect of *Nigella sativa* aqueous extract and chitosan nanoparticles against experimentally induced *Acanthamoeba* keratitis. Parasitol Res. 2019;118(8):2443–2454.